

R. Vanholder
 R. Dell'Aquila
 V. Jacobs
 A. Dhondt
 N. Veys
 M. A. Waterloos
 N. Van Landschoot
 W. Van Biesen
 S. Ringoir

Nephrology Department,
 University Hospital, Ghent, Belgium

Depressed Phagocytosis in Hemodialyzed Patients: In vivo and in vitro Mechanisms

Abstract

Infection is a frequent complication and the major cause of death among end-stage renal patients. Polymorphonuclear phagocytes (PMNL) are important in host defense mainly because of bacterial destruction by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-related free radical production following phagocytosis. In this study, hexose monophosphate pathway glycolytic activity, delivering energy to NADPH oxidase, is evaluated in vivo and in vitro, in healthy controls and in dialyzed renal failure patients. Our results show a marked parallel and correlated inhibition in the response to three stimuli for phagocytic activity (*Staphylococcus aureus*, formyl-methionine-leucine-phenylalanine, phorbol myristic acid) in predialysis samples. These data point to a main suppression of metabolic pathways, possibly beyond protein kinase C. This response is further suppressed at the 15th minute of cuprophane dialysis, for all stimuli studied (-40 to -94%; $p < 0.001$) except PMA. PMNL response remains intact during dialysis with non-complement-activating dialyzers. In vitro experiments confirm decreased PMNL glycolytic activity after the suspension of cuprophane fragments in normal whole blood. We conclude that polymorphonuclear cell energy delivery to NADPH oxidase is impaired in patients with end-stage renal failure. The impaired response against various stimuli is different in predialysis blood samples compared to samples collected during cuprophane dialysis, and may be related to two different conditions. These events probably contribute to the acquired immune suppression of uremia and the high incidence of infection among dialysis patients.

Key Words

Hemodialysis
 Bioincompatibility
 Cuprophane
 Infection
 Immune system
 Phagocytosis
 Polymorphs
 Uremia
 Uremic toxins

Introduction

Bacterial infection and septicemia account for approximately one fifth of the deaths in populations with end-stage renal disease, and are a major cause of morbidity and hospitalization [1]. Several factors may contribute to this defective host defense against infection. Phagocytosis by polymorphonuclear cells plays a critical role by ingestion of bacteria, and their subsequent destruction by bactericidal

mechanisms. Dysfunction of phagocytes thus will pose a threat to the host.

Although phagocytic function in uremic and dialyzed patients has been the subject of repeated evaluations, results have been contradictory [2-4], whereas the underlying metabolic changes were scarcely evaluated.

Bacterial destruction that follows phagocytosis largely depends on the production in the polymorphonuclear cell membrane of bactericidal oxygen-free radicals [5, 6].

Among those the generation of O_2^- is controlled by NAD(P)H oxidase, that is actuated directly by protein kinase C (PKC) in response to various cell membrane receptor and other activating mechanisms [7, 8]. The energy required for this process is delivered by the lysis of glucose, in the hexose monophosphate shunt (HMS), in the so-called respiratory burst. This metabolic pathway reflects the basic functional capacity of the phagocytes, but has not been the subject of extended study in dialyzed uremics.

In a recent study, we demonstrated an ex vivo disturbance of polymorphonuclear cell HMS-dependent glycolysis in renal patients [9]. A correlation was found between depressed glycolytic HMS activity and the chances to develop serious infection and/or septicemia. In the latter study, no definite conclusions about the basic mechanisms of this phagocytic defect were made, in vivo results were not matched to in vitro experiments, and no studies were performed to evaluate the intradialytic evolution of metabolism, with different stimuli, in relation to different dialysis membranes.

In the present study, we undertook a more extensive evaluation of the disrupted HMS activity in dialyzed uremics by determining the activation of PMNLs by five different well-known stimuli, either phagocytic [latex, zymosan, *Staphylococcus aureus*, (Staph A)] or humoral [N-formyl-methionine-leucine-phenylalanine (f-mlp) and phorbol myristic acid (PMA)]. The stimuli were chosen according to their capacity to activate phagocytosis via diverse pathways in the prospect to obtain suggestion about the mechanisms involved. Our data indicate that the defect of phagocyte metabolic glycolytic response is partly present in the predialysis status and partly enhanced acutely during dialysis due to the bioincompatibility of dialysis membranes and further suggest that both defects are the consequence of different mechanisms in the metabolic response during phagocytosis.

Materials and Methods

In vivo (Clinical) Studies

All patients participating in this study gave their informed consent. Exclusion criteria consisted of a history of autoimmune disorders, diabetes, malignancy, hematologic or liver disease, or of active or recent infections documented by hospitalization, leukocytosis or fever $> 38.5^\circ\text{C}$ in the past 3 months. Patients on antibiotics, corticosteroids or immunosuppressive medications were also excluded. All patients were on 1,25-vitamin D_3 and a serum level above 30 pg/ml was maintained. At the time of the present study, none of the patients was on erythropoietin. Patients with varying long-term duration times on dialysis (3 months to 10 years) were investigated. Dialysis occurred at blood flows of ± 300 ml/min and dialysate flows of ± 500 ml/min against bicarbonate dialysate.

Hemodialyzed Patients, Predialysis Samples. In a comparison to a population of 37 healthy controls, the metabolic response to phagocytosis in whole blood samples of 64 chronic hemodialysis patients was studied. All samples were obtained predialysis, at the same time as the routine monthly biochemical and hematological evaluation. All patients studied had had the same membrane and the same dialytic prescription for the previous 3 months. Three stimuli (Staph A, f-mlp, PMA) were used in, respectively, 24, 23 and 23 patients. Correlation-regression was analyzed, each time when at least 15 paired responses to two different stimuli were available.

Intradialytic Samples. To evaluate the acute effect of blood contact with dialysis membranes on phagocytic glycolytic function, in a prospective study, 14 stable chronic dialysis patients, currently treated with biocompatible, non-complement-activating dialyzer membranes, were treated consecutively and in random order during 4 consecutive weeks with four different dialyzer membranes: cuprophane as a reference membrane (Gambro 1.8, Lund, Sweden), containing cuprophane from ENKA (Wuppertal, FRG), compared to membranes with lower complement and/or leukocyte activation capabilities. These consisted of either high flux dialyzers containing synthetic polymethylmethacrylate (Toray B2, Tokyo, Japan) or polysulfone PS600 (F60) membranes (Fresenius, Bad Homburg, FRG), or low flux hemophane membranes (Gambro GFS 1.3). The high flux dialyzers were used in a hemodiafiltration setting, with 10 liters ultrafiltration and substitution by an isotonic solution. All dialyzers were ETO-sterilized.

Blood samples were collected at the last dialysis day of each treatment week, at the start of dialysis (0 min), after 15 min, and at the end of dialysis (240 min), from the inlet blood line. These studies were performed on whole blood with latex or zymosan as stimulants.

In addition, 10 patients were studied, only during treatment with first use cuprophane, for glycolytic reactivity of their PMNLs against latex, zymosan, f-mlp and PMA.

In vitro Studies

Effect of Dialyzer Membrane. The metabolic response of PMNLs was also evaluated in response to in vitro incubation with different dialysis membrane fragments (10 experiments). Using sterile techniques, fragments of cuprophane or polysulfone capillary membranes were obtained. The ratio of membrane to blood was approximately 50 mg of membrane (corresponding to approximately 60–80 cm^2 surface area) to 8 ml of heparinized blood. Incubation was allowed for 5, 10, 15, 30 and 60 min at 37°C in a shaker. An aliquot of the blood was then used to assess the metabolic response of the PMNLs to latex or zymosan. Control samples were processed in the same way, but without the addition of membrane fragments, and the difference in CO_2 response between the membrane and non-membrane-containing samples was calculated.

Methods: Glucose-1- ^{14}C Uptake

The principle of the assay for the determination of glucose breakdown has been described in detail in our previous papers [9–11]. In brief, glycolysis was evaluated by measuring $^{14}\text{CO}_2$ generated from the metabolism of glucose-1- ^{14}C by the HMS pathway, with and without incubation with latex, zymosan or Staph A particles as phagocytic challengers, or the soluble compounds f-mlp or PMA as humoral stimuli.

In a sterile capped tube, 50 μl of heparinized blood were mixed immediately after withdrawal with 10 μl containing 0.5 μg glucose-1- ^{14}C (Amersham, London, UK) and were incubated in a constant temperature 37°C shaker for 1 h. The $^{14}\text{CO}_2$ produced was continuously

trapped by a filter containing 100 μ l of 1 N NaOH. Metabolism was then arrested by cooling the tube to -20°C . For baseline studies (resting state), no additional stimulus was added, whereas the glycolytic activity in response to phagocytic challenge was evaluated by the addition of the stimuli as described below. At the end of the procedure, the NaOH-impregnated filter was removed, dried, and the $^{14}\text{CO}_2$ measured after addition of 5 ml Ultima Gold (Packard, Downers Grove, Ill.) in a β -counter and expressed as disintegrations per minute (dpm) and normalized for the number (in thousands) of PMNL determined separately by a Technikon analyzer. All analyses were performed in triplicate and all baseline and stimulated samples were processed simultaneously and in parallel. The results of this part of the study are reported as delta dpm/ 10^3 PMNL, which represents the difference in dpm (adjusted for cell count) between the baseline sample and dpm of samples after incubation with each specific stimulus.

Latex (Difco, Detroit, Mich.) was prepared as a stock solution of approximately 2.5×10^9 particles/ml (0.8 μm), whereas zymosan (Sigma, St. Louis, Mo.) was prepared as a stock solution of 5 g/100 ml, also resulting in approximately 2.5×10^9 particles/ml. Staph. A. (ATCC 6538P) was obtained after culture and repeated washing, in suspensions containing approximately 5×10^8 bacteria/ml. In preliminary studies, optimal concentration and time dependence were determined for the agonists by the maximum change in dpm between baseline and stimulated samples. The optimal concentrations were 1 PMNL to approximately 100 latex particles, 1 PMNL to 100 zymosan particles, and 1 PMNL to 20 staphylococci. The volume of the agonists was adjusted as close as possible to these ratios, depending on the leukocyte count.

F-mlp and PMA were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, Mo.) to obtain stock solutions at respective concentrations of 50 and 10 $\mu\text{g}/\text{ml}$, and in the glucose uptake experiments 10 μl were added to 50 μl of blood.

To obtain baseline values in the f-mlp and PMA experiments, DMSO without f-mlp or PMA was added to blood samples as a resting state experiment, and the increase in dpm/ 10^3 PMNL was calculated as the difference between the baseline values and those after stimulation, as described above.

In some experiments, opsonized and nonopsonized Staph A were compared. Opsonization was obtained by suspending these particles after centrifugation and withdrawal of the supernatant, in equivalent quantities of a pool of serum of healthy donors. After 30 min at 37°C in a shaking incubator, the particles were washed twice in isotonic saline, before their addition to blood samples.

Interference in the results by the presence of red blood cells, lymphocytes and thrombocytes in the whole blood samples under evaluation, or by shifts in pH and electrolyte or glucose concentration, in the ranges currently encountered in dialysis patients, was excluded in preliminary studies [9].

Statistics

Results are expressed as means \pm SEM. Statistical analysis of data for dialytic changes was performed by variance analysis (ANOVA). Data between different dialyzers and between different groups of patients or samples were first analyzed using variance analysis and when statistical significance was reached, the remaining sets of data were submitted to Wilcoxon's test for paired or unpaired values. The correlation-regression analyses were performed by Spearman's test. Statistical significance was accepted for $p < 0.05$.

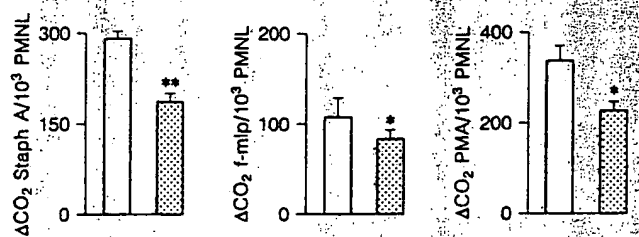


Fig. 1. Glycolytic response after challenge in healthy controls (□) and dialyzed renal failure patients, predialysis samples (▨). * $p < 0.01$; ** $p < 0.001$, vs. healthy controls.

Results

In vivo Studies

Hemodialyzed Patients, Predialysis Samples. The metabolic response to phagocytic (Staph A) and soluble stimuli (f-mlp, PMA) was evaluated on predialysis samples, and compared to samples obtained from healthy controls. For each of these stimuli the values of CO_2 production (177 ± 20 for Staph A, 78 ± 12 for f-mlp, 230 ± 18 dpm/ 10^3 PMNL for PMA) were lower than in healthy controls (fig. 1). For the matched responses towards different stimuli, a correlation-regression analysis each time showed a significant correlation [data not shown].

The study of opsonized versus nonopsonized Staph A and zymosan particles revealed no significant differences.

Hemodialyzed Patients, Intradialytic Samples. When different dialysis membranes were evaluated, the existing decrease in metabolic response of PMNL became even more pronounced during cuprophane dialysis (table 1). The nadir of glucose breakdown was registered at the 15th minute of cuprophane dialysis. The decrease versus predialysis was different for latex compared to zymosan (-69 and -35% , respectively, $p < 0.05$). In contrast, such a decrease in PMNL response appeared to be absent during dialysis with polymethylmethacrylate, polysulfone and hemophane (table 1). With the latter membranes, after 15 min, we even observed an increase in metabolic response ($p < 0.001$ vs. cuprophane at 15 min).

The decrease in PMNL metabolic response to stimuli at the 15th minute of cuprophane dialysis is further confirmed in a more elaborate study, comparing the stimuli, latex, zymosan, f-mlp and PMA (fig. 2). Note that the predialysis values are already markedly lower than those observed in healthy controls. A further reduction in the response to latex, zymosan and f-mlp is absent with the direct PKC

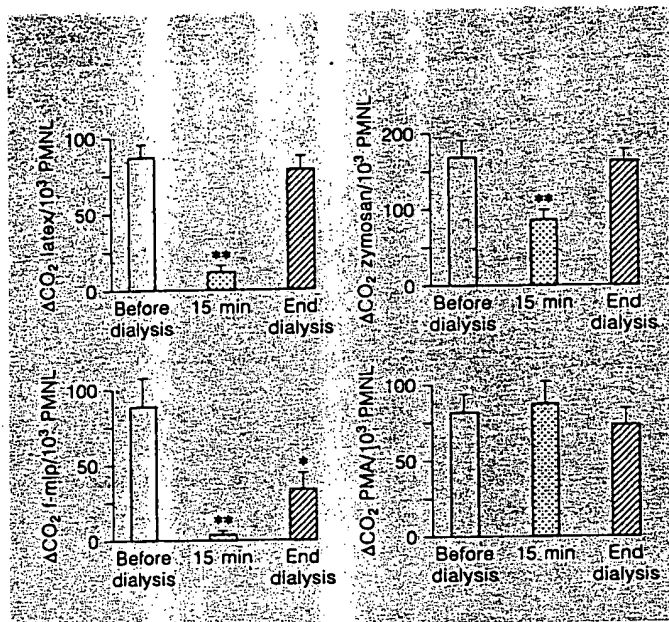


Fig. 2. Glycolytic response to various stimuli during cuprophane hemodialysis (n = 20). *p < 0.05; **p < 0.001, vs. predialysis.

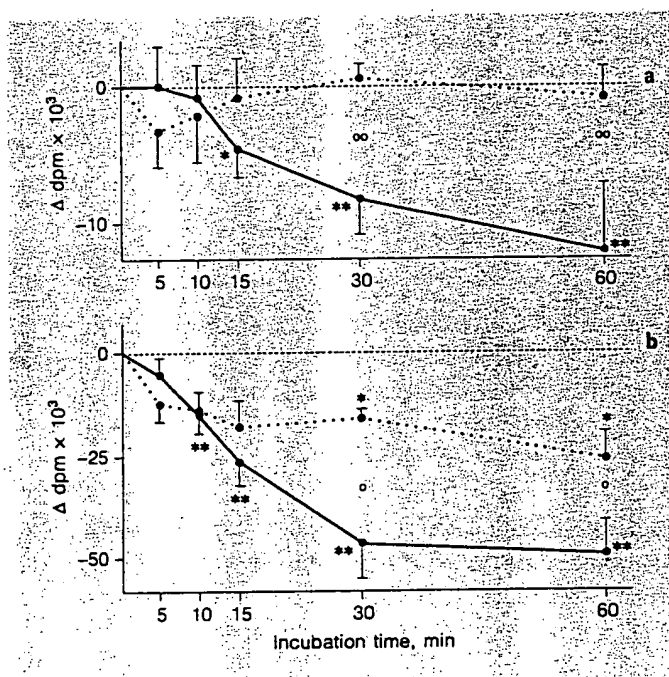


Fig. 3. Change in CO₂ production after addition to blood sample of cuprophane (—) or polysulfone membrane (---), compared to control samples with the same incubation time containing no membrane. Data expressed as the difference between membrane-containing and non-membrane-containing samples (Δ dpm), in response to latex (a) and zymosan (b). *p < 0.05; **p < 0.01, vs. incubation time of 0 min; °p < 0.05; °°p < 0.01, cuprophane vs. polysulfone.

Table 1. Intradialytic changes in PMNL metabolic response: cross-over study of cuprophane with non-complement-activating membranes (14 patients)

	Before dialysis	After 15 min of dialysis	End of dialysis
CO ₂ response to latex			
Cuprophane	71 ± 9	22 ± 8***	71 ± 12
Hemophane	96 ± 9	129 ± 11*°	100 ± 9
PMMA	93 ± 8	131 ± 11***°	98 ± 9
Polysulfone	97 ± 8	128 ± 9*°	96 ± 7
CO ₂ response to zymosan			
Cuprophane	187 ± 26	122 ± 21**	174 ± 27
Hemophane	170 ± 17	240 ± 20*°	188 ± 20
PMMA	196 ± 18	254 ± 18*°	185 ± 22
Polysulfone	189 ± 15	248 ± 15***°	187 ± 17

Data expressed as dpm/10³ PMNL.

*p < 0.05; **p < 0.01; ***p < 0.001, vs. before dialysis; °p < 0.001, vs. corresponding time point with cuprophane. PMMA = Polymethylmethacrylate.

Table 2. Mechanisms of granulocyte activation by the various stimuli used in the present study.

	Particle (phagocytosis)	Membrane receptors			Direct PKC activation
		IgG	C	other	
Latex	+	-	-	-	-
Zymosan	+	+	+	+	-
Staph A	+	+	-	-	-
f-mlp	-	-	-	+	-
PMA	-	-	-	-	+

IgG = Immunoglobulins; C = complement.

activator PMA. The percentage changes compared to predialysis at the 15th minute are markedly different (latex: -91%, zymosan: -49%, f-mlp: -94% and PMA: +6%). No intermutual correlation could be found.

In addition to the decrease seen at 15 min, the response remains suppressed compared to predialysis at the end of dialysis by 61% for f-mlp (p < 0.05). Thus, the decrease in glycolytic response seen in predialysis samples of dialyzed patients is further accentuated only during dialysis with cuprophane. This is however not the case for all stimuli, as the response against PMA remains preserved. In addition those changes are absent during dialysis with non-complement-activating membranes.

In vitro Studies: Effect of Dialyzer Membrane

As shown in figure 3, blood incubated with cuprophane membrane exhibits a decreased response to incubation with latex or zymosan. In contrast, blood incubated with polysulfone particles for comparable periods of time shows no change in $^{14}\text{CO}_2$ response to latex and a significantly more vigorous response to zymosan than samples incubated with cuprophane membrane.

Discussion

Phagocytic HMS shunt glycolytic activity during the respiratory burst is affected in predialysis blood samples. A supplementary defect is observed during cuprophane hemodialysis. The first change may be related to retention of uremic factors, the second to bioincompatibility of dialysis membranes. The latter mechanism is underscored *in vitro*, showing suppression of HMS glycolysis after contact of whole blood of healthy donors with cuprophane fragments (fig. 3). *In vitro* studies on this subject up to the present mainly examined free radical production by evaluation of chemiluminescence [12–16]. However, a decrease in chemiluminescence production in azotemic blood samples not necessarily points to an intrinsic metabolic defect of PMNLs, as chemiluminescence in fact reflects oxidative free-radical production. The latter can be neutralized by antioxidants, and in this respect it should be stressed that uremic serum is rich in scavenger molecules (phenols, indoles, uric acid) [17–19]; it thus remains a matter of debate whether a decrease in chemiluminescence reflects a decrease in metabolic activity or antioxidant action of retained solutes. In contrast, in the present study, we demonstrate changes in metabolic activity *per se*.

Although the present data do not exclude a carryover of defective function from previous dialyses, in view of the lack of improvement at the end of dialysis, there are other data from our group that suggest that toxic factors are involved: (1) PMNL function is depressed in outpatients already before the start of dialysis [9], and (2) PMNL functional disturbances can be induced by suspension of normal PMNLs in uremic plasma and ultrafiltrate [20].

In many studies on this subject [and also in our own previous study, 9] only a limited number of stimuli are evaluated and/or compared, which interferes with the interpretation of underlying mechanisms. The five stimuli used in the present study were chosen in regard of their different mechanisms of polymorphonuclear cell stimulation (table 2), in the hope of shedding more light on the

processes involved in effete PMNL function in end-stage renal disease. Latex, zymosan and Staph A are particulate 'phagocytic' stimuli, but the mechanisms involved are different for each of them. Latex, after its engulfment due to cell surface charge interactions, stimulates HMS activity via direct intracellular mechanisms [21]. Zymosan combines with membrane receptors for IgG, C3b, mannose and β -glucan [22–24]. Staph A acts via membrane/bacteria interactions involving opsonization of cell wall components [25]. F-mfp is a nonparticulate soluble ('chemotactic') agent, but induces stimulation by surface membrane receptor mechanisms [26]. PMA is a soluble stimulator by direct translocation and activation of PKC [27], bypassing plasma membrane-mediated receptors. PKC in turn activates NADPH oxidase directly to generate oxygen-free radicals [7]. Parallelism in the response towards all these different stimuli points away from any mechanism that is not common to all these stimuli together. The mechanisms involved follow more or less common pathways from the level beyond PKC; therefore, if all stimuli react in parallel, deactivation beyond the level of PKC can be supposed to be involved.

This paper should thus be considered as a further elaboration of our previous work [9], in order to answer the following questions. (1) Do changes occur in parallel to all stimuli and is there an intermutual correlation? (2) Can changes be attributed to mechanisms involving receptor expression and particle ingestion, or are they mainly due to metabolic alterations? (3) Is there a difference in the pattern involved before dialysis versus during dialysis? (4) Are changes during dialysis different depending on the dialyzer membrane used? (5) Can *in vivo* changes be reproduced *in vitro*?

In addition, whereas in the previous study emphasis was put on chronic changes in response to the dialysis membrane, the present study was mainly designed to evaluate the predialysis condition and acute intradialytic changes.

Before dialysis, a parallel suppression is found in the response against all three stimuli, including direct PKC activator PMA (fig. 1). Consequently, we propose a functional disturbance of the metabolic pathways as key mechanism for this defect, as even the reaction towards direct PKC-activating PMA is reduced. The questions concerning the responsible pathophysiologic mechanisms remain unanswered. Chemical compounds may inhibit respiratory burst and/or NADPH oxidase activity [28, 29], and we cannot exclude the possibility that a similar inhibition occurs by some of the manifold solutes retained in uremia, but to our knowledge NADPH oxidase activity has as yet not been studied in relation to uremia and/or uremic compounds.

An additional decrease in the response to three of four stimuli was particularly remarkable after 15 min of cuprophane dialysis (fig. 2). Interestingly reactivity to PMA was maintained. Reactivity remained suppressed at the end of dialysis for f-mlp. These data are difficult to compare with other data in the literature, because of differences in the technical approach, but there is a striking analogy to the recent findings by Himmelfarb et al. [30], Hirabayashi et al. [31] and Roccatello et al. [15], each time with one of the five stimuli used in the present study (f-mlp, PMA and zymosan, respectively). Our observations of a dissimilar response to various stimuli suggest that the suppression intradialysis is controlled by a different mechanism than that involved before dialysis, where all responses are similarly depressed. As PMA is the only stimulus that is strong enough to overcome this suppression in response, it is more difficult to accept here a disturbance of the metabolic axis involving HMS, PKC and/or NADPH oxidase. It is also worth noting that for zymosan the suppression is markedly less pronounced.

Our data conform with acute infection, where there is a poor response to various stimuli of PMNL function [32]. In infection, this effect is attributed to complement activation products [33], which are also abundant during cuprophane dialysis [34–36]. Neutrophils preexposed to high concentrations of activated complement are inhibited in their subsequent migratory responses [37]. However, this deactivation is mainly related to locomotion and not to NADPH oxidase activity [38].

Alternative explanations may be considered. First, neutrophils activated during passage through the dialyzer are sequestered in the pulmonary vasculature on their return to the circulation [35], and the immunologically most reactive cells are more prone to take part in this process. This probably leads to an increase in the proportion of functionally effete cells with a reduced response that remain in the bloodstream [39]. Second, polymorphonuclear cell deactivation of HMS response may be related to the release of other humoral compounds, e.g. tumor necrosis factor [40], which has been found recently in increased concentrations during cuprophane dialysis [41].

Note that this effete response intradialysis should be discerned from the chronic decrease in predialysis activity during prolonged treatment with cuprophane dialysis, which we described previously [9]. Both effects may be additive and may subsequently induce dramatic changes in the PMNL response towards infection, not only in chronic maintenance dialysis patients, but also in intermittent dialysis, e.g. for acute renal failure.

We conclude that the polymorphonuclear cell HMS response to stimuli is decisively disturbed in dialyzed patients. This intrinsic metabolic defect is related to two different conditions (predialysis and during cuprophane hemodialysis), with a discordant response towards various stimuli. The reduction in host response mechanisms in uremia may explain the propensity to infection seen in patients with severe renal failure and dialysis, and also indicates that both renal failure and dialysis may have systemic actions.

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